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Neurotrophic protein S100β stimulates glial cell proliferation

(mitogen/platelet-derived growth factor/protooncogene/astrocyte/mutagenesis)

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ABSTRACT **Nervous system development involves a coordinated series of events, including regulation of cell proliferation and differentiation by specific extracellular factors. S100β is a neurotrophic protein that has been implicated in regulation of cellular proliferation, but direct evidence was lacking. In this report, nanomolar concentrations of S100β are shown to stimulate proliferation of rat C6 glioma cells and primary astrocytes. An S100 mutant with a single amino acid change was inactive. S100β also stimulated increases in the steady-state levels of c-*myc* and c-*fos* protooncogene mRNAs and complemented the effects of platelet-derived growth factor. Two neuroblastoma cell lines did not proliferate in response to S100β, suggesting that the mitogenic activity of S100β is selective for astroglial cells. These results suggest that S100β may be involved in the coordinate development and maintenance of the central nervous system by synchronously stimulating the differentiation of neurons and the proliferation of astroglia.**

S100β is a small, acidic, calcium binding protein that is in highest concentration in the vertebrate nervous system. The protein is found primarily in astroglial cells in the central nervous system (CNS) and Schwann cells in the peripheral nervous system (1–3). S100β is present in lower concentrations in a number of tissues outside the nervous system, including adipose tissue, testis, and skin (4–6). S100β shares amino acid sequence homology with a family of proteins, some of which have been implicated in regulation of cell growth and differentiation events (7–19). The gene for human S100β maps to the Down syndrome region of chromosome 21 (20, 21). In addition, S100β levels are elevated in reactive glial cells of patients with Down syndrome and Alzheimer disease (22, 23), suggesting a potential involvement in the neuropathologies, and possibly the memory loss and mental retardations, associated with these disorders.

Although the exact *in vivo* function of S100β is not known, increasing evidence suggests an extracellular role. S100β has been detected in brain extracellular fluid and in conditioned medium from astroglial cells (24–26). The extracellular levels of S100β are highest during exponential growth of the glial cells, whereas the intracellular levels of the protein accumulate when the cells are confluent (26–28). Extracellular S100β appears to act as a trophic factor for certain neuronal populations. Specifically, a disulfide-linked dimeric form of S100β enhances survival of embryonic chicken cortical neurons in culture (29) and stimulates neuritic outgrowth from cortical neurons (29–31), serotonergic neurons of the mesencephalic raphe (32), optic tectum (33), spinal cord (34), and dorsal root ganglia (35) neurons and from the Neuro-2a cell line (36).

In addition to the neurotrophic activity of S100β on neuronal cells, indirect evidence has suggested that S100β contributes to glial cell proliferation. For example, using antisense approaches, we have recently shown (37) that selective inhibition of S100β production in glioma cells reduces cell growth rate. Additionally, it has been suggested that S100β levels in glioma cells are linked to particular phases of (38), or progression through (39), the cell cycle. Furthermore, S100β levels are elevated during the period of glial cell proliferation and neuronal differentiation (40–42). These observations have led to suggestions that S100β may influence development of the CNS by stimulating the differentiation of neurons and the proliferation of glial cells. At high concentrations, a mixture of S100α and S100β proteins stimulates proliferation of melanoma cells and lymphocytes (43). However, evidence for a direct effect of S100β on glial cell proliferation has not been reported. Therefore, we tested the ability of S100β to stimulate proliferation of a glial tumor cell line (rat C6 glioma cells) and rat primary astrocytes. As a control, we tested a mutant S100 protein (C68V) that has previously been shown (29) to lack neurotrophic activity on cortical neurons. We report here that S100β has mitogenic activity on both glial cell types, whereas the C68V mutant containing a single amino acid change is not mitogenic for these cells.

MATERIALS AND METHODS

Protein Purification. Recombinant S100β (VUSB-1) and the C68V mutant were expressed by synthetic genes in *Escherichia coli* and purified as described (29, 31). Further purification to apparent homogeneity was done by fast preparative liquid chromatography on immobilized W-7 [*N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide]. W-7 was coupled to cyanogen bromide-activated Sepharose 4B, and columns were run essentially as described (44). After elution from the W-7 column with buffer containing 1 mM EGTA, S100β and C68V were stored at −20°C in the presence of 4 mM added calcium. We have found that the effective dose of S100β varies somewhat, depending on the conditions of storage of the protein. For example, when S100β is stored at −20°C in the presence of a chelator, the biological activity is less than that seen with S100β preparations stored in the presence of calcium. Although the precise reason has not been determined, preliminary evidence suggests this may be related to the enhanced ability of S100β to form dimers in the presence of calcium.

Cell Culture. Rat C6 glioma cells (American Type Culture Collection) were routinely maintained in alpha-minimal essential medium (α-MEM; GIBCO) supplemented with 2.5% (vol/vol) fetal bovine serum (FBS; HyClone), 20 units of penicillin per ml, and 20 μg of streptomycin per ml (GIBCO). Primary astrocytes were prepared from neonatal rat pups (ages <24 hr) as described (45), except that cells were plated at 5×10^4 cells per cm^2 in α-MEM containing 15% FBS. After 24 hr, cells were washed six times with serum-free α-MEM to remove loosely attached cells. Cells were routinely main-

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Abbreviations: CNS, central nervous system; FBS, fetal bovine serum; PDGF, platelet-derived growth factor.

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tained in α-MEM containing 10% FBS and used before passage 6. Neuroblastoma cells [B104 (46) and Neuro-2a (American Type Culture Collection)] were maintained in α-MEM containing 10% FBS.

Proliferation Assays. For analysis of S100β mitogenic activity, C6 cells were placed into 2-cm^2 tissue culture wells at a density of 5000 cells per well and allowed to grow overnight in the presence of 2.5% FBS. Cells were then washed twice with serum-free α-MEM and incubated for 48 hr in isoleucine-deficient α-MEM containing 0.05% FBS to allow the cells to become quiescent. Cells were stimulated by changing the medium to α-MEM containing 0.05% FBS alone (control) or 0.05% FBS plus various concentrations of S100β, C68V, platelet-derived growth factor BB (PDGF-BB; Upstate Biotechnology, Lake Placid, NY), or insulin (Collaborative Research). At the time of stimulation (time 0) and at 24-hr intervals thereafter, the medium was removed from some of the wells. The cells were suspended by incubation in phosphate-buffered saline (PBS) containing 0.125% trypsin and 0.5 mM EDTA, diluted into isotonic saline, and counted on a Coulter Counter (Coulter). Duplicate counts were performed on duplicate dilutions from triplicate wells for each condition. Mean cell number ± SEM is expressed as a percentage of the control cells at each time. Primary astrocytes and neuroblastoma cells were assayed for proliferation exactly as described above, except that cells were plated at a density of 10,000 cells per well and growth-arrested in α-MEM containing 0.125% FBS (astrocytes) or 0.2% FBS (neuroblastoma cells).

For analysis of [^{3}H]thymidine incorporation, primary astrocytes were placed into 0.32-cm^2 tissue culture wells at a density of 8000 cells per well in α-MEM containing 10% FBS. At 2 days after confluency (total culture period = 7 days), the density-arrested cells were stimulated by changing the medium to serum-free α-MEM containing 10 μg of bovine serum albumin per ml (fatty acid-free, Miles), 5 μCi of [^{3}H]thymidine per ml (5 Ci/mmol; 1 Ci = 37 GBq; Amersham), and S100β. At the indicated times after stimulation, the cells were washed twice in PBS, fixed in two washes of 100% methanol, and washed eight times with water. Cells were solubilized in 0.1 ml of 0.2 M NaOH/1% sodium dodecyl sulfate, transferred with an additional 0.1-ml wash of the respective well into Beckman Ready-Safe scintillation cocktail, and assayed for ^{3}H.

Northern Blot Analysis. Subconfluent cultures of C6 glioma cells were growth-arrested for 48 hr in α-MEM containing 0.05% FBS and then stimulated by the addition of S100β to the cells without a medium change. At the indicated times after stimulation, cells were lysed and total RNA was isolated as described (47). Electrophoresis, Northern blotting, and hybridization were performed as described (48). The c-*myc* probe was a 4.8-kilobase (kb) *Xba* I–*Bam*HI fragment of pSVc-myc1 (49), and the c-*fos* probe was a linearized pSP70-fos (50). Probes were labeled with [^{32}P]dCTP using a random-prime labeling kit (Boehringer Mannheim). Equal loading of RNA was confirmed by ethidium bromide staining of the rRNA.

RESULTS AND DISCUSSION

The ability of S100β to stimulate proliferation of astroglial cells was tested. The mitogenic effects of S100β were assayed by standard methods used for testing the possibility that a protein can function as a growth factor (for review, see ref. 51). Briefly, cells are made quiescent by either low serum arrest or density arrest, and then the ability of a growth factor to stimulate proliferation is ascertained by measurement of increases in cell number and/or [^{3}H]thymidine incorporation. As shown in Fig. 1, addition of S100β to quiescent cultures of C6 glioma cells resulted in stimulation of cell proliferation in a time- and dose-dependent manner. S100β was effective in the nanomolar concentration range, with the maximal growth-stimulatory effect in this experiment being 3 nM (30 ng/ml). Similar effects of S100β were seen in primary astrocytes from neonatal rats, although slightly higher concentrations of S100β were generally required (data not shown). The data in Fig. 1 were obtained with a recombinant S100β produced in *E. coli*. However, we have also found that a commercial source of S100 (East Acres Biologicals, Southbridge, MA) has activity, although it is at least 30- to 100-fold less active than our preparations.

To determine the specificity of the S100β-induced proliferative response, we tested a mutant S100β protein that has a single cysteine to valine change at residue 68. This mutant, termed C68V, has been shown (29) to be inactive in neurite extension assays. We found that C68V did not stimulate C6 glioma cell proliferation (Fig. 1). In fact, high concentrations of C68V appeared to inhibit the growth rate. The reason for the inhibition of growth at high concentrations of C68V was not pursued as part of these studies, although it is interesting to speculate that C68V may be acting in competition with possible autocrine effects of endogenous S100β released from

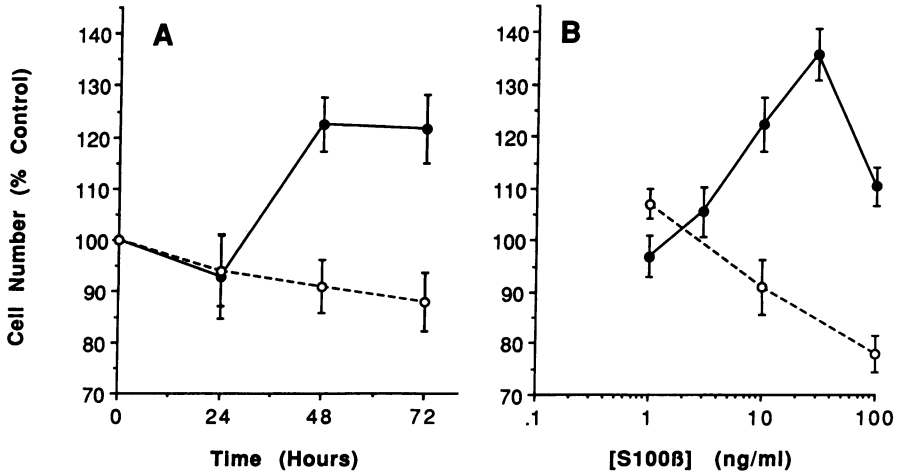

FIG. 1. S100β stimulates glial cell proliferation. Subconfluent rat C6 glioma cells were made quiescent in low serum for 48 hr. Cells were then stimulated by changing the media to α-MEM containing 0.05% FBS alone (control) or 0.05% FBS plus various concentrations of S100β (●) or C68V (○). Cell numbers were determined at the time of stimulation (time 0) and at 24-hr intervals thereafter. Mean cell number ± SEM is expressed as a percentage of the control cells at each time. (*A*) Time course of growth in the presence of 10 ng of S100β or C68V per ml (14). (*B*) Dose dependence of the effects at 48 hr after stimulation.

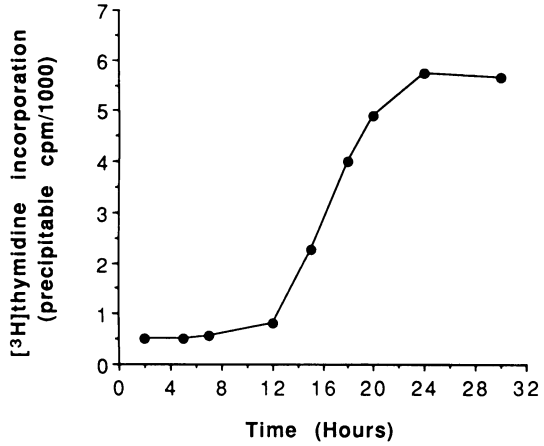

FIG. 2. S100β stimulation of [^{3}H]thymidine incorporation. Confluent, density-arrested rat primary astrocytes (passage 4) were stimulated by changing the medium to serum-free α-MEM containing bovine serum albumin, [^{3}H]thymidine, and 10 or 30 ng of S100β per ml (the specific activity of S100β varied with storage time; see text). At the indicated times after stimulation, cells were solubilized and incorporated cpm were determined. Data represent the mean of two experiments for which triplicate determinations were made. The standard deviation between 24-hr values in the two experiments was ±495 cpm.

the cell. The data presented in Fig. 1 demonstrate that the S100β molecule is a growth factor for glial cells, and the inactivity of C68V suggests that the mitogenic activity of S100β may be dependent on some of the same structural requirements as its neurotrophic activity.

To further examine the S100β-induced growth response, we measured the ability of S100β to stimulate increases in DNA synthesis. As shown in Fig. 2, addition of S100β to postconfluent, density-arrested cultures of primary astrocytes resulted in an increase in [^{3}H]thymidine incorporation, occurring 12–16 hr after S100β addition. Autoradiographic assessment of the number of cells with thymidine-labeled nuclei confirmed the increase in DNA synthesis (data not shown).

We tested the ability of other serum growth factors to complement S100β in stimulating cellular proliferation. It is known that multiple growth factors present in serum stimulate growth synergistically (52) and that insulin and PDGF can stimulate glial cell growth (53–55). The simultaneous

Table 1. Cell type selectivity of S100β mitogenic activity

Cell type	Cell number* Negative control	S100β	Stimulation,† % control
C6 glioma	6,504 ± 271	8,850 ± 318	136
Primary astrocytes	16,470 ± 356	20,940 ± 995	127
Neuro-2a	13,038 ± 1590	6,750 ± 327	52
B104	24,540 ± 794	24,360 ± 952	99

*Cell numbers were determined at 48 hr after change to medium alone (negative control) or medium with 30 ng of S100β per ml. Duplicate counts were performed on duplicate dilutions from duplicate wells for each condition. Values shown are mean cell number ± SEM.

†Stimulation is expressed as a percentage of the negative control values for each cell type. Stimulation of the C6 glioma ($P < 0.01$) and primary astrocytes ($P < 0.05$) by S100β was significantly above control, and cell numbers in the S100β-treated Neuro-2a cells were significantly below control ($P < 0.02$), as determined by a two-tailed test of significance (56).

addition of S100β and PDGF to primary astrocytes, at concentrations at which each added individually had little effect on cell growth above controls, resulted in a significant and apparently synergistic stimulation of proliferation (Fig. 3*A*). In contrast, the simultaneous addition of S100β and insulin yielded an essentially additive effect on proliferation (Fig. 3*B*).

We also examined the generality of S100β's mitogenic activity for various cell types. As demonstrated in this report, S100β stimulates proliferation of both C6 glioma cells and primary astrocytes. However, two neuronal cell lines were not stimulated to proliferate in response to a wide range of S100β concentrations. As shown in Table 1, growth-arrested rat neuroblastoma B104 cells were not stimulated to divide by S100β, and mouse neuroblastoma Neuro-2a cells were actually inhibited by concentrations of S100β that stimulate glial cell proliferation. To address the possibility that the lack of responsiveness of the neuroblastoma cells to S100β reflected a corequirement for PDGF, we tested S100β effects in the presence or absence of PDGF. It is possible that C6 cells and primary astrocytes produce PDGF or another growth factor that then acts in concert with S100β to stimulate glial cell proliferation. In this regard, our preliminary studies indicate that S100β can stimulate proliferation of growth-arrested BALB/c-3T3 A31 cells only in the presence of PDGF (un-

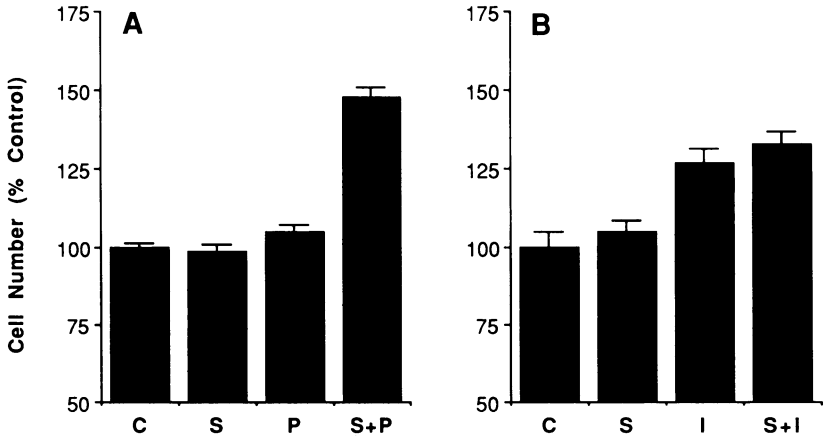

FIG. 3. Effects of PDGF or insulin on S100β mitogenic activity. Primary astrocytes (passage 5) were made quiescent in low serum for 48 hr. (*A*) Cells were stimulated with medium containing 0.125% FBS alone (control, C) or 0.125% FBS plus 10 ng of S100β per ml (S), 1 ng of PDGF per ml (P), or both proteins (S+P). (*B*) In a separate experiment, cells were stimulated with medium containing 0.125% FBS alone (control, C) or 0.125% FBS plus 10 ng of S100β per ml (S), 10 μg of insulin per ml (I), or both proteins (S+I). Data reflect mean cell number ± SEM as a percentage of the control cells at 48 hr after stimulation. Duplicate counts were performed on duplicate dilutions from duplicate wells for each condition.

published observations). However, simultaneous addition of S100β and PDGF did not stimulate proliferation of the neuroblastoma cell lines above the stimulation by PDGF alone, suggesting that neuronal cells are not a target for the mitogenic activity of S100β. Interestingly, Neuro-2a cells have been reported (36) to extend neurites in the presence of S100β, a response consistent with the induction of differentiation rather than proliferation.

The mechanism by which S100β stimulates cell proliferation is unknown, but many growth factors are known to induce protooncogenes like c-*fos* and c-*myc* (57). Therefore, we tested by Northern blot analysis the ability of S100β to induce c-*myc* and c-*fos* mRNA levels in growth-arrested C6 cells. As shown in Fig. 4, S100β stimulated a transient increase in the steady-state mRNA levels for c-*myc* that was maximal at 2 hr after S100β addition (Fig. 4*A*) and a transient increase in c-*fos* mRNA levels that was maximal at 30 min after S100β addition (Fig. 4*B*). The ability of S100β to induce the mRNAs for these cellular protooncogenes suggests that S100β stimulates cell proliferation through a mechanism similar to that proposed for other serum-derived growth factors, at least at the level of protooncogene expression.

The data reported here, that demonstrate mitogenic activity of S100β for glial cells, combined with the previously documented neurotrophic activity of S100β for neuronal cells and the developmental expression of S100β during the period of neuronal maturation and glial cell proliferation, suggest an important role for S100β in CNS development. Taken together, the available evidence suggests a model whereby S100β helps coordinate development of the CNS by synchronously stimulating the differentiation of neurons and the proliferation of astroglia. Precedence for this model includes other trophic factors having the dual function of stimulating differentiation and proliferation. Some relevant examples are transforming growth factor-β (58), γ-interferon (59, 60), fibroblast growth factor (61), and a heparin binding protein, termed pleiotrophin (62), that has mitogenic and neurite outgrowth activity. Confirmation of a dual role for S100β will require elucidation of the mechanisms that enable neurons and astroglia to respond differentially to S100β. Studies to determine whether S100β utilizes conventional signal transduction pathways including binding of cell surface receptors, stimulation of second messengers, and control of gene expression are necessary.

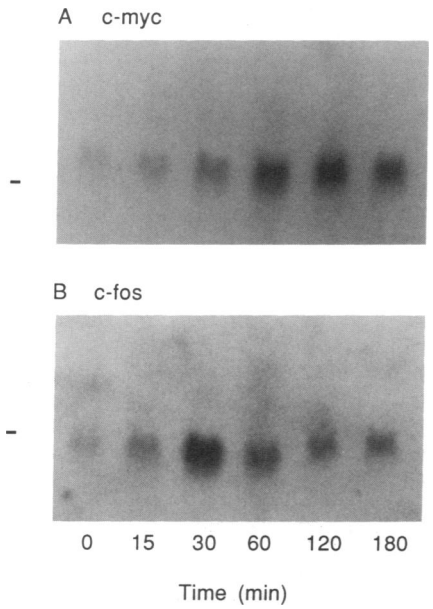

FIG. 4. Stimulation of protooncogene mRNAs by S100β. Rat C6 glioma cells were made quiescent in low serum for 48 hr in α-MEM containing 0.05% FBS and then stimulated by the addition of S100β to the cells without a medium change. At the indicated times after stimulation, cells were lysed and total RNA was isolated. Equal amounts of RNA (7 μg per lane) were subjected to Northern blot analysis by probing the blot successively with probes to c-*myc* (*A*) and c-*fos* (*B*). The position of 18S rRNA is indicated at the left.

Based on S100β action on glial and neuronal cells, it is clear that aberrant S100β gene expression and protein production/targeting during critical periods of development could have profound effects on nervous system function. Perhaps the abnormally high levels of S100β present during development in the brains of patients with Down syndrome (22) are linked to the neuropathologies associated with this disorder. The marked increase in S100β mRNA, protein, and neurite extension activity in Alzheimer disease (23) suggest that elevation of S100β may also be linked to the neuropathological events that culminate in this disorder. Our demonstrations that S100β can affect glial cell morphology (37) and proliferation also suggest that the protein may contribute to the gliosis in Down syndrome and Alzheimer disease.

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